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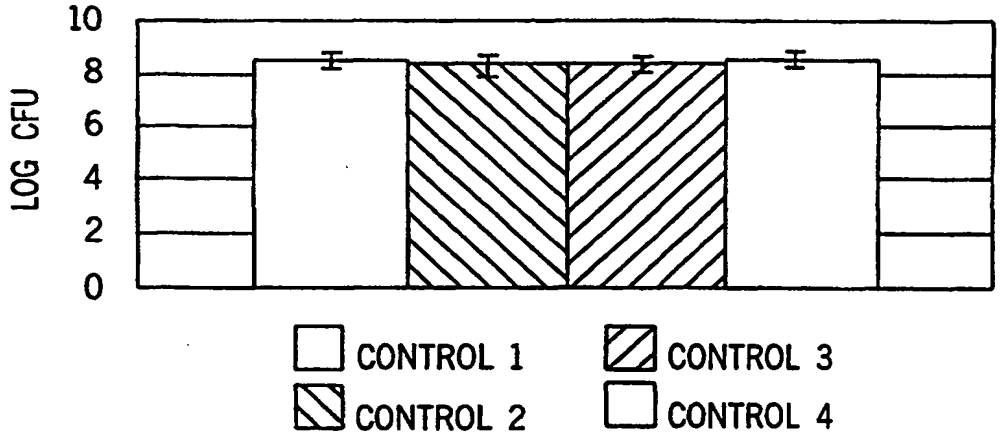
(54) Title: A MODEL BIOFILM FOR EFFICACY ASSESSMENT OF ANTIMICROBIALS



(57) Abstract: A method for growing a model biofilm is disclosed. In one embodiment, the method comprises the steps of placing a plurality of surfaces comprising a top and a bottom on an inoculated growth medium (wherein the growth medium is in contact with a nutrative source), growing a model biofilm on the bottom of the plurality of surfaces, and removing the surfaces from the growth medium, wherein a model biofilm is coated onto the bottom of the surface.

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A MODEL BIOFILM FOR EFFICACY ASSESSMENT OF ANTIMICROBIALS

Cross-Reference To Related Application

This application claims priority to U.S. Provisional Application, Serial No. 60/138,354, filed June 10, 1999.

Statement Regarding Federally Sponsored Research Or Development

Not applicable.

BACKGROUND ART

Microorganisms, whether in homes, manufacturing plants, soil, water, or a human body naturally tend to grow as biofilms (Raloff, 1996; Blackman and Frank, 1996; Olson, 1997; Potera, 1999). Biofilm cells are phenotypically different from planktonics (Characklis, 1990; Gilbert, 1990; Costerton, et al., 1995). One of the most important manifestations of this difference is the significantly decreased susceptibility of biofilm cells to biocides (Costerton, et al., 1995; Das, et al., 1997).

Disinfectant registration protocols are based on the acceptance that microorganisms grown suspended in liquid laboratory cultures are generally representative of those found in the environment. However, the target organisms used to measure the efficacy of antimicrobials in the laboratory are not always identical to the organisms causing problems. By not taking into consideration microorganisms growing as biofilms when testing the efficacy of antimicrobials, current test protocols may not adequately measure all aspects of disinfectancy.

One straightforward approach to include biofilm testing in a disinfectant testing regime would be to substitute biofilm covered carriers for those covered with planktonic cells as used in current tests approved by the Association of Official Analytical Chemists (AOAC). Biofilm-covered coupons suitable for disinfectancy testing can be grown in a variety of biofilm reactors. However, these methods

require expertise and the use of expensive equipment and produce a limited number of available test coupons.

Attempts have been made to reduce the complexity of laboratory grown biofilms in order to simplify biocide susceptibility testing. Cells may be entrapped into an artificial matrix such as alginate (Stewart, et al., 1997; Xu, et al., 1996), other polymeric material (Härkönen, et al., 1998), or adsorbed onto alginate beads (Cochran, 1997). There is evidence that these entrapped cells show increased resistance to biocides that are independent of transport limitations imposed by the alginate matrix (Cochran, 1997). However, the system is somewhat artificial in that cells are not embedded in their own extracellular polymeric substance (EPS). In addition, these artificial biofilms are intrinsically different from the test surfaces utilized in current methods of disinfectant testing and difficult to substitute into a current test protocol.

DISCLOSURE OF INVENTION

I have developed a model biofilm that is reliable, simple to prepare and not dependant on expensive equipment. In brief, the model biofilm is grown on coupons, or other planar surfaces that are placed on inoculated growth media, such as filter paper, on a nutrative support, such as agar. These biofilm-covered coupons may be substituted for the carriers covered with planktonic cells that are used in conventional biocide testing. The model biofilm is a naturally grown biofilm and the cells are not placed in an artificial environment. The model biofilm is neither "reactor grown" nor grown at a solid/liquid interface, as are most test biofilms. The biofilm of the present invention is grown under controlled conditions and is reproducible.

One embodiment of the invention comprises the steps of placing a plurality of surfaces on an inoculated growth medium (wherein the growth medium contacts a nutrative source), growing a model biofilm on the bottom of the plurality of surfaces, and removing the surfaces from the growth medium. The surfaces will be covered with the model biofilm of the present invention.

It is an object of the present invention to provide a plurality of reproducible test surfaces covered with model biofilm for use in testing protocols.

It is another object of the present invention to provide a rapid, reproducible, and low cost method for creation of model biofilm test surfaces.

Other objects, advantages and features of the present invention will become apparent after examination of the specification, claims and abstract.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 is a schematic of one embodiment of a model biofilm set up.

Fig. 2 is a bar graph recording the number of cells recovered from different control runs.

Fig. 3 is a bar graph describing the effect of hypochlorites on model biofilms of the present invention.

Fig. 4 is a bar graph describing the effect of test products on biofilm.

Fig. 5 is a bar graph tabulating the effect of two test biocides on three different types of microorganisms, e.g. *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Klebsiella pneumoniae*.

Fig. 6 is a bar graph comparing changes in inoculum and nutrient availability in a *Staphylococcus aureus* model biofilm.

Fig. 7 is a bar graph comparing cell numbers recovered from model biofilm of the present invention versus reactor grown biofilm.

DETAILED DESCRIPTION OF THE INVENTION

I have developed a method for growing a model biofilm in response to the need for a relatively realistic, simple, and reliable test for biocide efficacy. This method utilizes basic laboratory supplies and it is fast (48 hours), simple and reproducible. Moreover, the method does not limit the availability or number of test coupons. One could easily have hundreds of coupons available with relatively minimal effort.

The cells of the biofilm of the present invention are embedded in EPS that the cells produce. I prefer to categorize this biofilm as a 'model' because I do not know that the cells have undergone all of the prerequisite phenotypic changes attributed to a true biofilm.

The model biofilm does have some of the characteristics of wild biofilms. For example, the cells adhere to glass slides or other test surfaces, produce slime, and show significantly increased resistance to antimicrobial agents. Preferably, attached cells with visible slime production are in a range of 10^3 to 10^{12} cells per cm^2. The preferred range is 10^7-10^8. It is preferable that the solid coupon does not directly touch a solid, non-porous surface. Data obtained with such a model biofilm may be validated against those obtained from natural or reactor-grown biofilm samples that are treated identically.

Method for preparing model biofilm

In one embodiment, disclosed in Fig. 1, the method involves growing biofilm 2 on an inoculated growth medium 4, preferably filter paper (Whatman qualitative #2), placed on top of a nutrative source 6, preferably agar, e.g. Trypticase Soy Agar. I used 10 x 10 cm square Petri dishes with 40 ml agar per plate.

Suitable nutrative sources are those that can support a porous sheet. This could be a liquid medium with a frame or other structure supporting the filter paper or a sponge-like sheet saturated with medium (i.e., the porous support could be thick enough to make the agar underneath unnecessary). Therefore, the nutrative source and the inoculated growth medium may be the same physical structure.

A quantity, usually 1 ml, of a diluted (1/10 to 1/100) overnight culture of a desired biofilm-forming organism is pipetted onto the filter paper so that the entire paper surface is evenly moistened. Filter paper is porous, and its purpose is to reduce the possibility of anoxic conditions developing on the underside of the coupons.

Many other porous growth media, as mentioned above, may be suitable. It is only necessary that the suitable growth media support the growth of the test organisms.

Referring to Fig. 1, a set of planar surfaces 8, preferably sterile flat coupons (e.g. glass or stainless steel), are placed on top of the inoculated filter paper 4, and lightly pressed down. As the biofilm 2 grows on the surface of the filter paper 4, the biofilm 2 also covers the underside of the surfaces 8. It is helpful to re-moisten the filter paper, preferably after 24 hours with either dilute (1/100) Trypticase Soy Broth

or phosphate buffered saline. The biofilm-covered coupons are ready to be harvested when the biofilm is mature, preferably after 48 hours.

Typically, when I harvest the coupons I clearly see a slimy film growth on the filter paper between the coupons. I have looked at the coupons after one day and found biofilm to be present. I preferred to let the model biofilm grow for two days, however, to ensure biofilm maturity, i.e. complete physical and physiological transformation of the attached cells to biofilm. I found little difference in response and cell numbers in 1 to 4 day old samples. A suitable time period would give the cells time of for attachment and biofilm formation and may take as long as two weeks. Preferred time is 2 - 3 days. Another embodiment of the invention would be to grow up the biofilm on the chelated support for some time and then place the coupons on top of the established biofilm. Cells will adhere to the coupons within a short time, even minutes. This may be less reproducible.

I grow the cultures for the inoculation of the model biofilm depending on the preferred growth conditions of the respective organism, i.e., *Pseudomonas aeruginosa* (environmental strain PAO1) at ambient room temperature, and *Staphylococcus aureaus* (ATTC 6538), a clinical strain, at 35 ±2°C. The model biofilm, however, I prefer to grow at ambient room temperatures irrespective of the type of cells used.

Preferably, the coupons are aseptically removed from the surface of the filter paper with a forceps and used either immediately or after drying for 40 minutes at 35±2°C. The drying step corresponds to the prior art preparation of planktonic carriers.

The coupons are then subjected to biocide testing, preferably as outlined in AOAC Official Methods of Analysis.

EXAMPLES

A. Methods to evaluate model biofilm

I evaluated the reproducibility of the model biofilm growth by scraping and enumerating >20 untreated coupons. I consistently obtained about 10^8 cells/coupon with variability within one log (see Fig. 2). There was no difference between using

coupons immediately after harvesting or dried coupons (40 minutes at 35±2°C). Neutralizing broth did not adversely affect the cells.

To better distinguish cells from extracellular polymeric substance (EPS), cells and EPS were differentially stained following procedures developed by Allison and Sutherland, 1984 and D.G. Davies, 1999. Alcian blue was used to stain the EPS blue and carbol fuchsin to stain the cells a contrasting red.

B. Biocide testing

1. Testing of planktonics, qualitative. Each active ingredient was first tested on planktonic cells following the procedure outlined in the AOAC Germicidal Spray test. A cell suspension was applied to flat glass coupons, 6.44 cm^2 in size. The coupons were dried for 40 minutes at 35±2°C and sprayed with a biocidal treatment. After an exposure time of 10 minutes, the coupons were transferred to a neutralizing broth, incubated for 48 hours and observed for growth. According to the AOAC Official Method, an active or product passes the disinfectancy test if no more than 1 out of 60 tubes shows growth due to surviving cells.
2. Testing of biofilm, qualitative. Model biofilm coupons of the same size and prepared by the method described above were substituted for the planktonic carriers and tested by the AOAC method described above.
3. Testing of biofilm, quantitative. Five or more biofilm coupons were exposed to each biocidal treatment for a contact time of 10 minutes or a suitable shorter or longer time depending on the usage and type of biocide used. The biofilms were then removed from the carriers by scraping directly into the neutralizing broth. (This is the first dilution step.) The cells were then dispersed by homogenization for 1 minute at ½ maximal speed after 24 to 48 hours. The cell suspensions were then serially diluted and plated on R2A or other suitable agar. R2A media is supplied by BBL or Difco (DF1826-17-1), included in Handbook of Microbiological Media, Ronald M. Atlas, CRC Press, 1993, ed. Lawrence C. Parks. Cells surviving the treatment were counted as CFU's after 24 to 48 hours of incubation. An equal number of untreated coupons was also scraped into the neutralizing broth and processed

identically to the treatments. These served as controls used to calculate log reductions by a method developed at the Center for Biofilm Engineering at Montana State University (Hamilton and Herigstad, 1998).

C. Results/Discussion

The advantage of this model biofilm is its ease of preparation and reproducibility between samples. The cell density of a 48-hour model biofilm consistently reached about 10^8 cells/coupon with variability within 1 log (Fig. 2). The model biofilm can easily be seen with the naked eye as a slimy material adhering to the underside of the coupons. Microscopic examination by brightfield microscopy shows cell clusters associated with EPS. In fact, our model biofilm is indistinguishable from stained reactor grown biofilm, although confocal microscopy may reveal differences in biofilm architecture.

Biocidal efficacy testing enables the evaluation and ranking of biocidal products. Biofilm coupons were substituted for the planktonic preparations in the AOAC Germicidal Spray Test and other standard antimicrobial test to obtain a qualitative assessment of the disinfectant efficacy of products versus biofilm. To facilitate the comparison, initial cell numbers on biofilm and planktonic test coupons were comparable, i.e. 10^8 to 10^9 cells per coupon (10^6 to 10^7 cells/cm^2 of coupon surface). I believe that the results thus obtained are in range with results described in the literature for artificial biofilms (Chen and Stewart, 1996) as well as for environmental or reactor grown biofilms (Samrakandi, et al., 1997).

Table 1 shows the results for the qualitative AOAC Germicidal Spray tests for *Pseudomonas aeruginosa* PAO1 and *Staphylococcus aureus* 6538 planktonic cells and biofilm. All tests involving planktonics consistently passed the AOAC Germicidal Spray Test, i.e. there were no surviving cells on any of the 60 coupons that had been treated. In contrast, none of the identically treated biofilm samples passed.

	Pseudomonas aeruginosa		*Staphylococcus aureus*	
Biocidal treatment, *Passing = (1/60 positive*	**Planktonic**	**Biofilm**	**Planktonic**	**Biofilm**
1000ppm NaOCl	0/60	60/60	0/60	39/60*
1000ppm NaOCl formula #1	0/60	59/60	0/60	60/60
1000ppm NaOCl formula #2	0/60	60/60	0/60	60/60
Product #1	0/60	43/60	0/60	
Product #2	0/60	43/60	0/60	
Product #3	0/60	19/60	0/60	
Product #4	0/60	32/60	0/60	

Table 1: AOAC qualitative Germicidal Spray Test: Effect of biocides on planktonic cells and biofilm.

*These samples may have been insufficiently neutralized.

Producing 60 or more coupons for the qualitative test is not difficult and the main effort of the test lies in the treatment step. However, using fewer coupons and the more labor-intensive enumerative process yields results that can be evaluated statistically. With the quantitative evaluation it is possible to rank the efficacy of biocides versus biofilm. The 1000 ppm hypochlorite treatment of the model biofilm resulted in a roughly 2-log reduction of biofilm cells. Hypochlorite test formulation #1 proved to be less effective than the hypochlorite standard but proprietary formula #2 proved to be the most effective of all actives and products tested (Fig. 3). As

expected, the four consumer products tested were less effective against biofilm than indicated by standard test methods based on planktonics (Fig.4).

I also tested *Staphylococcus aureus* (ATCC 6538), *Klebsiella pneumoniae* and *Enterobacter aerogenes* against 2 standard test biocides (see Fig. 5). Although *Staphylococcus aureus* showed slightly higher kill in response to both biocides than *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Enterobacter aerogenes* (not included in graph), almost identically showed reduction of cells of only approximately 1 log or less. It may be advantageous to eventually tailor biofilm composition for test applications according to the predominant species found in the respective environment. Little is known about the synergistic or antagonistic actions of mixed biofilms with respect to biocides and the method of the present invention may be a suitable tool for this evaluation.

D. Effect of cell inoculum and nutrient concentration on model biofilm growth.

I examined inocula of varying cell dilutions varying cell dilutions and varying nutrient concentrations on model biofilm growth. The model biofilms were harvested after 42 to 48 hours of incubation. Table 2 and Table 3 tabulate the cells per coupon resulting from a particular cell dilution/nutrient concentration.

Table 2

Staphylococcus aureus, cells per coupon of model biofilm					
Cell Dilutions	**TSA 100%**	**TSA 50%**	**TSA 25%**	**TSA 10%**	**TSA 1%**
1:1			7.96E+06	4.10E+07	7.56E+05
1:10	9.31E+07	7.70E=07	1.37E+07		7.78E+05
1:100	2.23E+08				
1:1000	3.05E+08				
1:1000,000	2.41E+08		2.09E+07	4.32E+07	0

Table 3

Klebsiella pneumoniae, cells per coupon of model biofilm					
Cell Dilutions	**TSA 100%**	**TSA 50%**	**TSA 25%**	**TSA 10%**	**TSA 1%**
1:1				2.69E+07	
1:10	2.13E+08			5.51E+06	
1:100					
1:1000					
1:1000,000				8.56E+05	

Fig. 6 is a bar graph describing the results with the *Staphylococcus aureus* model. For *Staphylococcus aureus* decreasing the number of cells placed on the porous medium did not change the number of biofilm cells harvested after 48 hours. Decreasing the nutrient concentration of the agar only affected the number of biofilm cells significantly below 5% (i.e. at 1%) of the original nutrient concentration. For *Klebsiella pneumoniae* a similar trend was observed. (Empty boxes represent experiments not done because a trend can be established from the available data.)

E. Presence of polysaccharides in model biofilm.

The model biofilm described above was exposed to two stains: Alcian Blue for polysaccharide and Carbol Fuchsin for cells. I stained all cell types, i.e. *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*. All preparations showed cells associated with EPS. The clustering effect was most prominent for *Staphylococcus aureus*, which has a natural trend to form microcolonies quickly. In this way, the polysaccharide was stained

blue and the cells red. I showed clearly that polysaccharide was present and that it was closely associated with the cells. In particular, *Staphylococcus aureus* model biofilm cells were observed to cluster into microcolonies, such biofilm cells are described in the literature.

F. Comparison of model biofilm with reactor-grown biofilm.

Growing biofilms in annular reactors has widely been described in the literature. Annular reactors (RotoTorque) were produced by Biosurface Technologies, Inc. in Bozeman, MT (Biofilms, John Wiley & Sons, William G. Chakaraklis and Kevin C. Mashall, pp. 59-62, 1990). I had a modified RotoTorque build in which the long stainless steel coupons on which biofilms are grown were replaced by holders accepting 4 glass coupons each. These glass coupons are identical to those in size used for the model biofilm. Comparison of cell numbers recovered from model biofilm and the modified TotoTorque are comparable.

Table 4 and Fig. 7 describe the comparison of model biofilm with reactor-grown biofilm. This treatment data show that the model biofilm reacts to the standard treatments, i.e. 1000 ppm NaOCl and 1000 ppm Quat + 200 ppm EDTA, in a manner similar to reactor-grown biofilm.

Table 4

	Modified RotoTorque	**Model Biofilm**
Untreated control	1.70E+07	3.82E+07
1000 ppm NaOCl	5.00E+05	3.13E+05
1000 ppm Quat+200 ppm EDTA	1.44E+06	1.44E+06

INDUSTRIAL APPLICABILITY

The modle biofilm of the present invention provides a multitude of reproducible test surfaces for use in testing protocols. Preparing the modle anti-microbial biofilm involves relatively inexpensive equip0ment and materials and is not limited by the number of test coupons. The method is fast (48 hours), simple and reproducible.

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Claims

I claim:

1. A method of growing a model biofilm comprising the steps of

a. placing a plurality of surfaces comprising a top and a bottom on an inoculated growth medium, wherein the growth medium is in contact with a nutrative source and wherein a model biofilm is allowed to grow on the growth medium,

b. allowing the model biofilm to attach to the bottom of the plurality of surfaces, and

c. removing the surfaces from the growth medium, wherein a model biofilm is coated onto the bottom of the surface.

2. The method of claim 1 wherein the growth medium is filter paper.

3. The method of claim 1 wherein the nutrative source is agar.

4. The method of claim 1 wherein the surface is a coupon.

5. The method of claim 4 wherein the coupon comprises glass or stainless steel.

6. The method of claim 1 wherein the number of surfaces is greater than 2.

7. The method of claim 1 wherein the surfaces are dried after removal.

8. The method of claim 1 wherein the surfaces are subjected to biocide testing after the removal of step (c).

9. The method of claim 1 wherein the model biofilm comprises a mixture of microorganisms or species.

10. The method of claim 1 wherein the number of surfaces is greater than 10.

11. A plurality of surfaces covered on at least one side with a model biofilm.

12. The method of claim 1 wherein the inoculated growth medium and the nutrative source are combined.

13. The method of claim 1 wherein the biofilm is allowed to grow before the surfaces are placed on the growth medium.

14. The method of claim 1 wherein the biofilm is allowed to grow after the surfaces are placed in the growth medium.

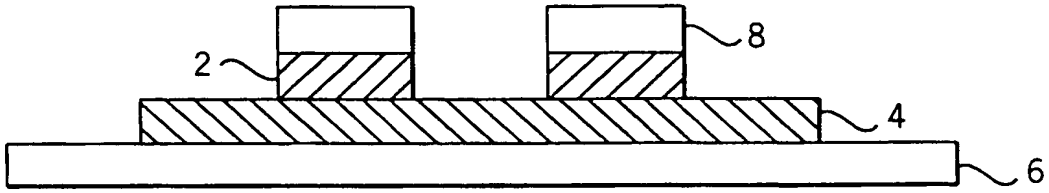


FIG. 1

FIG. 2

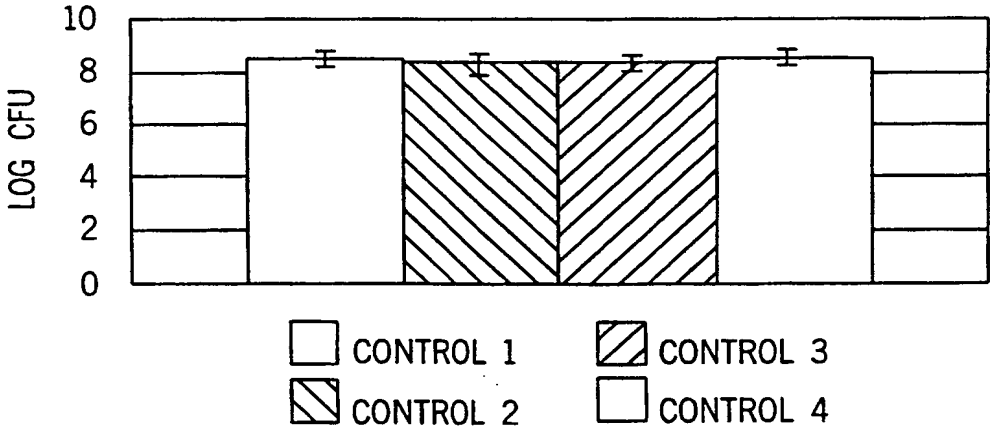
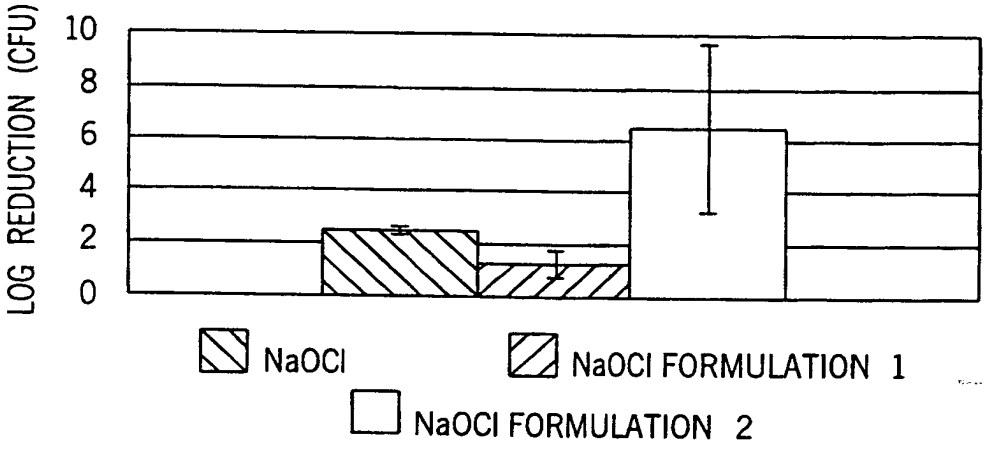


FIG. 3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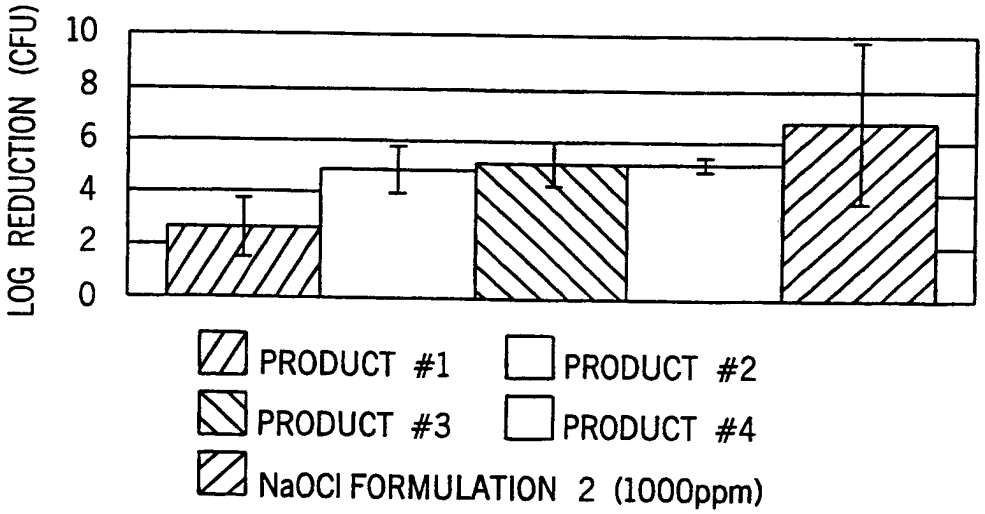
NaOCl
NaOCl FORMULATION 1
NaOCl FORMULATION 2

FIG. 4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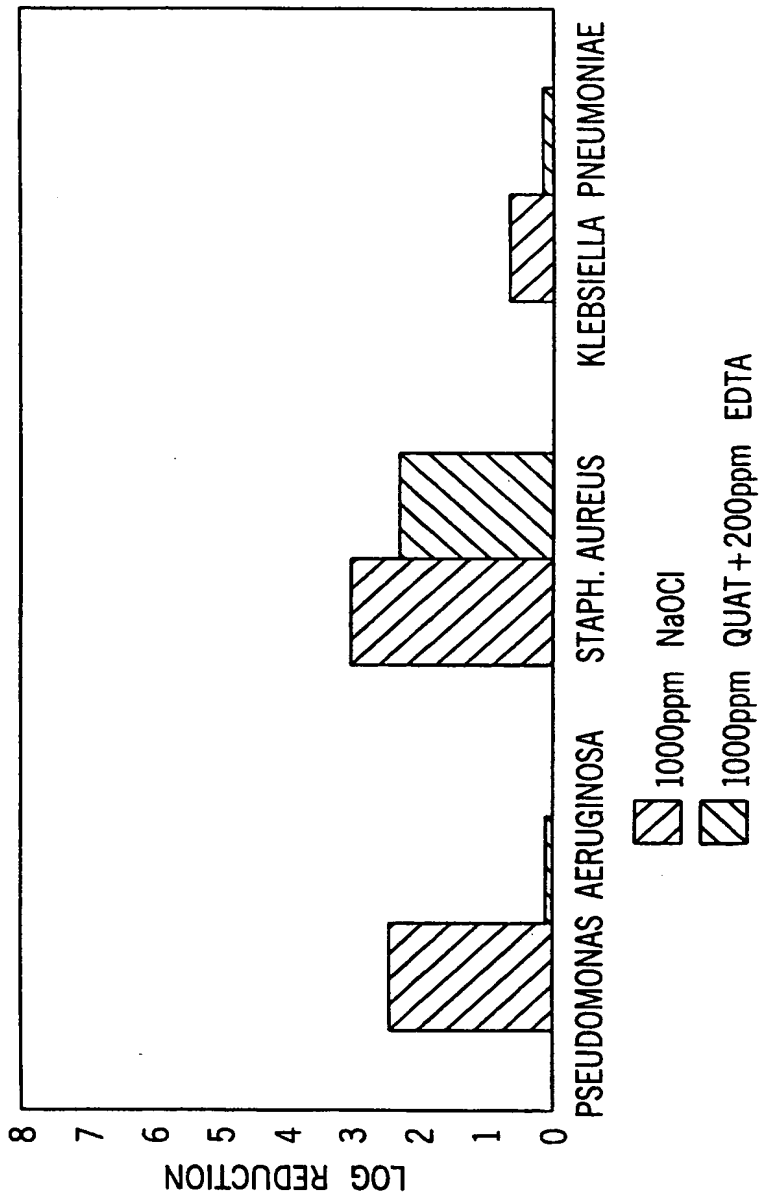
PRODUCT #1
PRODUCT #2
PRODUCT #3
PRODUCT #4
NaOCl FORMULATION 2 (1000ppm)

FIG. 5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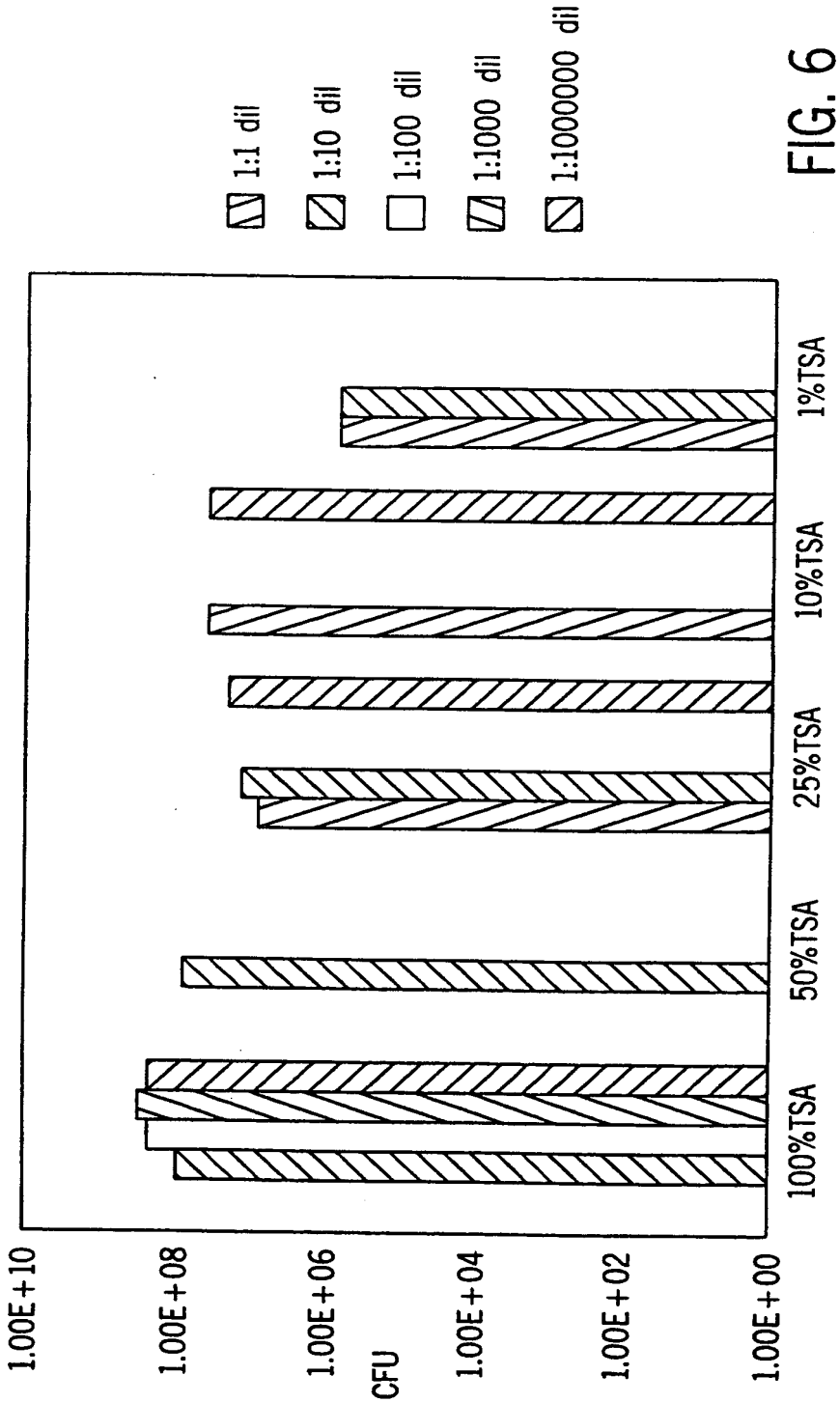
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1%TSA

FIG. 6

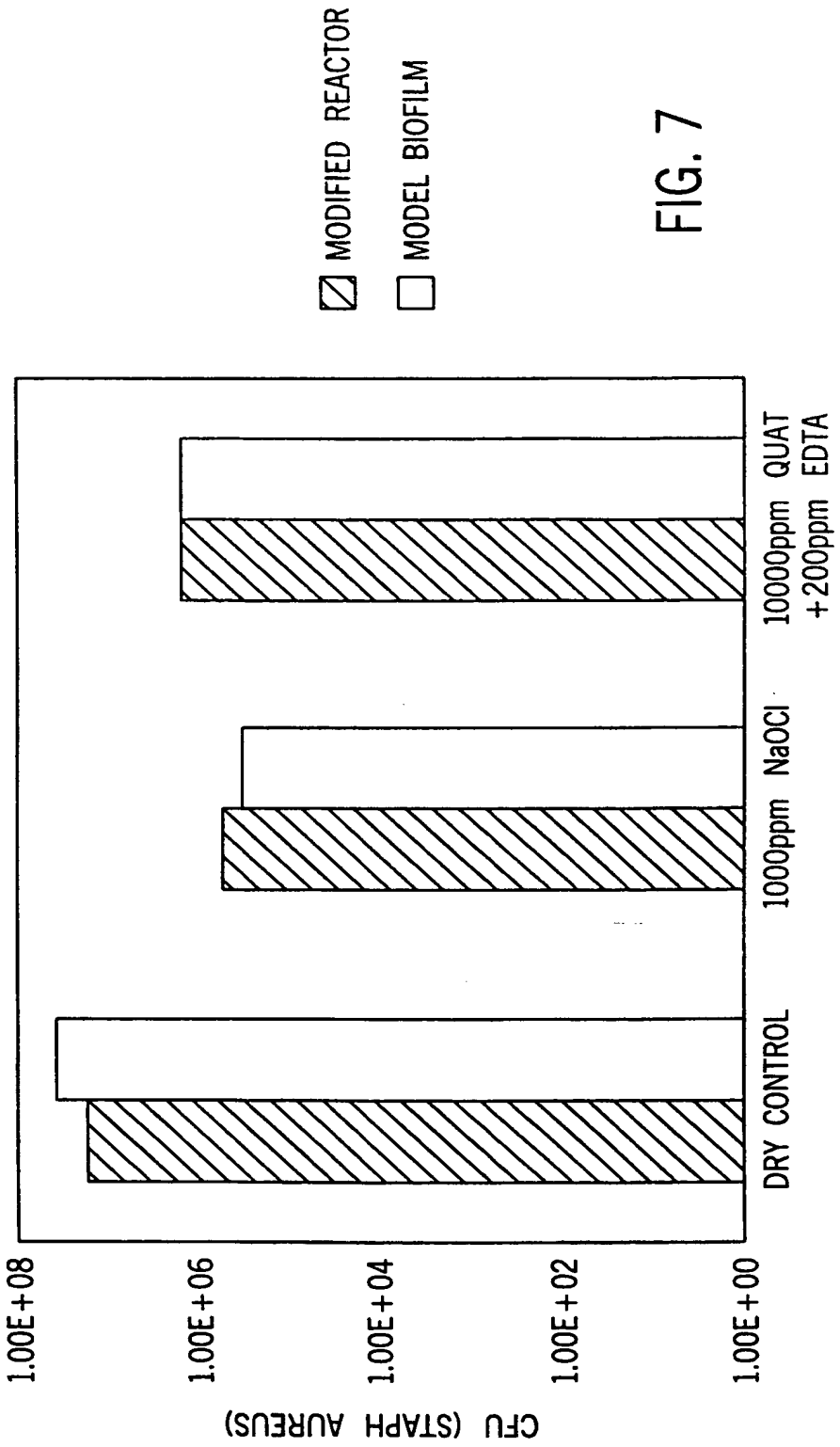
MODIFIED REACTOR
MODEL BIOFILM
CFU (STAPH AUREUS)
1.00E+08
1.00E+06
1.00E+04
1.00E+02
1.00E+00
DRY CONTROL
1000ppm NaOCl
10000ppm QUAT
+200ppm EDTA

FIG. 7

INTERNATIONAL SEARCH REPORT

Inter ›nal Application No
PCT/US 00/15675

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12M1/26

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 91 01364 A (IMP CANCER RES TECH) 7 February 1991 (1991-02-07) page 5, line 4 - line 21; claims; figures	1,3,5,6, 9-13
X	WO 95 27039 A (KEMIRA CHEMICALS OY) 12 October 1995 (1995-10-12) claims; figure	11
X	US 4 125 436 A (LINER JOHN) 14 November 1978 (1978-11-14) column 3, paragraph 3; claims; figures column 1, line 7	1,3,5,6, 9-12,14

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
13 November 2000	20/11/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Coucke, A

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte. onal Application No
PCT/US 00/15675

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